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PRINCIPAL INVESTIGATOR: Jian Zhang, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06511

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**6. AUTHOR(S)**
Jian Zhang, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Yale University
New Haven, Connecticut 06511

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One of the most alterations in human breast cancer is the overexpression of the c-erbB-2 proto-oncogene that has been shown to occur in 25-30% of human breast tumors. Recently this increased expression has been shown to correlate with the presence of the transcription factor AP-2. We have begun to determine the role of AP-2 both in normal mammary gland development and in breast cancer. we have examined the occurrence of AP-2 in human breast tumor biopsies and found that it is present in the majority of cases (80-90%). We have also found that c-erbB-2 expression is always associated with the presence of AP-2 in breast cancer biopsies. These data again support a role for AP-2 in controlling gene expression during breast cancer. In addition we have determined the differential regulation of AP-2 during the normal development of mouse mammary gland. We are now directly testing the role of AP-2 in breast cancer using animal model systems. In particular, we have established genetically altered mice to study the changes in breast biology associated with the aberrant expression of AP-2. We are also in process of testing if interaction between AP-2 and the c-erbB-2 gene can lead to breast cancer. Taken together, these studies will provide insight into the etiology of breast cancer and allow an assessment of the value and feasibility of using AP-2 as a target for gene therapy.

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## Introduction

Breast cancer is one of the most common and important diseases affecting women in North America and Western Europe (1, 3). Despite advances in treatment, only modest increases in survival have been achieved. Examination of the molecular changes in human breast cancer has revealed that one of the most common alterations is the overexpression of the normal c-erbB-2 gene. The human c-erbB-2 gene, which is normally expressed at low levels in a variety of adult epithelial cells, is overexpressed in 25-30% of carcinomas of the breast (2). Moreover, high levels of c-erbB-2 expression have been shown to correlate with poor prognosis and to predict a worse response to therapy (2, 3).

The normal c-erbB-2 gene (also known as *neu* or HER2) encodes a 185 kDa tyrosine kinase transmembrane receptor which shows extensive structural similarity with the epidermal growth factor receptor (17, 18). The relationship between c-erbB-2 and tumorigenesis was first noted when this gene was identified as the causative agent of chemically induced rat neuroglioblatomas (19). This rat oncogene contained a single base pair mutation, resulting in an amino acid change in the transmembrane domain of the protein. This alteration leads to autophosphorylation of the cytoplasmic portion of the protein which renders c-erB-2 constitutively active and oncogenic (19,20). This change is unlikely to occur in the human population because of codon usage differences; the same amino acid change in the human gene would require the unlikely occurrence of mutations in two adjacent base pairs. In fact, in a number of human tumors examined so far no equivalent mutation to the rat activated *neu* gene has been found (21-23). Indeed, the involvement of c-erbB-2 in human cancers always seems to involve a different mechanism, namely the overexpression of the normal gene (see below).

The potential of the c-erbB-2 gene to cause tumors has been assessed in both cultured cells (4, 5) and transgenic mice (6, 7). In tissue culture it has been shown that the normal human c-erbB-2 cDNA was able to induce transformation of NIH3T3 cells when fused to strong viral transcriptional control elements. Transgenic mouse experiments were first performed using the oncogenic version of the rat c-erbB-2 gene, *neu*, placed under the control of the mouse mammary tumor virus (MMTV) LTR to direct its expression to breast tissue. These transgenic mice gave rise to adenocarcinomas encompassing the entire mammary gland with extremely short latency (6). More recently, these experiments have been recapitulated using the normal version of the rodent *neu* gene under the control of the MMTV LTR. Transgenic mice carrying this construct developed focal mammary tumors after long latency periods that metastasized at high frequency (7). Thus, inappropriate overexpression of the normal *neu* gene in mammary tissue can induce breast carcinoma, supporting a role for this gene in human disease.

One of the fundamental questions arising from these studies is what is the mechanism of c-erbB-2 overexpression in human mammary carcinomas? In general the increase in c-erbB-2 mRNA and protein is associated with amplification of the gene (2),

although it can also occur from a single copy gene (8, 9,16). Indeed, studies in cell lines derived from human mammary tumors have shown that there is a 6-8 fold increase of c-erbB-2 mRNA per template copy in overexpressing cells, whether or not the gene is amplified (8, 9). The critical region of the human c-erbB-2 promoter that is responsible for the activation in overexpressing mammary cancer cells has recently been identified (11). Transfection and DNaseI footprinting assays have led to the identification of a transcription factor, originally termed OB2-1, present in c-erbB-2 overexpressing cells, that binds and activates the c-erbB-2 promoter (10). More recent studies involving our laboratory have shown that OB2-1 is antigenetically and functionally indistinguishable from the developmentally regulated transcription factor AP-2 (11-14).

The 50 kDa AP-2 protein was initially isolated from human Hela cells by virtue of its interaction with an important GC-rich recognition sequence present in the SV40 and human metallothionein IIa (hMtIIa) enhancers (24, 26). It has been shown that AP-2 is able to bind to cis-regulatory sequences present in a variety of viral and cellular genes, including proenkephalin, collagenase, TNFα, growth hormone, HTLV-1, and MHC class I H-2K$^b$ (24-28, 33). More recent reports have also implicated AP-2 in the transcriptional control of the estrogen receptor, hsp27, and epithelial cadherin (34-36). AP-2 is a sequence-specific DNA binding transcription factor that has a modular organization (13, 14). The N-terminus of the protein contains a region rich in proline and aromatic amino acid residues that serves as an activation domain both in the context of AP-2 or when attached to a heterologous DNA binding domain. The C-terminal half of AP-2 is responsible for sequence-specific DNA binding. This DNA binding portion consists of a basic region necessary for DNA contact and a large, stable dimerization domain of novel structure, termed a helix-span-helix (13, 14).

In the context of c-erbB-2, the critical promoter sequence required for high level expression in mammary tumor cell lines occurs approximately 200 nts upstream of the major RNA start site (10). This sequence, GCTGCAGGC, is closely related to the AP-2 consensus sequence, GCCNNNGGC (13). Functional assays confirmed that AP-2 was able to bind to this sequence and regulate c-erbB-2 expression in mammary derived cell lines (11). In co-transfection assays, the wild type AP-2 protein was able to stimulate c-erbB-2 promoter driven expression in mammary cells lacking AP-2. In contrast, a dominant negative version of AP-2 that only contained the dimerization domain was able to reduce expression in cell lines containing endogenous AP-2 protein. Finally, tumor cell lines containing high levels of c-erbB-2 were found to have high levels of AP-2 protein (11). Taken together, these studies indicate that AP-2 is a critical regulatory molecule in mammalian development and strongly suggest that it may play an important role in human breast cancer.

Further examination of the OB2-1 complex indicated that it contained other proteins in addition to AP-2. In particular, peptide sequence derived from OB2-1 demonstrated that it is actually a mixture of three proteins belonging to the AP-2 gene family (37). In addition to original AP-2 gene, other two novel AP-2 genes present in the OB2-1 complex have been isolated. A comparison of the predicted peptide sequence reveals that these new genes, AP-2 β and AP-2 γ, are highly related to the original AP-2

protein, now termed AP-2 α. The greatest degree of conservation is observed in the regions of the protein originally shown to be important for transcriptional activity. In particular, the DNA binding and dimerization domains are very well conserved (75-85%). Consistent with this finding, the three proteins all recognize the same DNA sequence and can form either homo- or hetero- dimers with themselves or with each other (37). Furthermore, the three AP-2 proteins are all capable of activating expression of reporter genes driven by the human c-erbB-2 promoter (37). Taken together, these data indicate that the c-erbB-2 gene can act as a target for transactivation by AP-2 proteins.

We and others have recently established the critical importance of AP-2 as a developmental regulator in murine embryogenesis. The AP-2 null-mice have severe defects in many organs including the head, brain, peripheral nerves, limbs, and the ventral body wall - where the mammary gland normally resides (38, 39). In contrast, little is known about the role of AP-2 in adult mouse development. Because AP-2 is a fundamental gene regulator and is associated with breast cancer, we have begun to determine the role of AP-2 both in normal development of mammary gland and in breast cancer. Since the award started on July 1, 1996, I have made excellent progress in pursuit of my specific aims. In particular, as outlined in more detail below, I have generated the majority of the transgenic animals which I had previously proposed. I have established genetically altered mice to study the changes in breast biology associated with the aberrant expression of AP-2. These mice will enable us to test the involvement of AP-2 in mammary gland development and its potential to induce mammary tumors either alone or in combination with c-erbB-2. I have also examined the occurrence of AP-2 in human breast tumor biopsies and correlated these data to the pattern of c-erbB-2 expression. I have also determined the differential regulation of AP-2 during the normal development of the mouse mammary gland. The data we have generated so far strongly support a role for AP-2 in normal development of mammary gland and in breast cancer.

**Materials and Methods**

**Tissue Collection**

Human breast cancer biopsies were obtained from Yale School of Medicine. These biopsies have already been analyzed in terms of patient history, including such criteria as age, tumor recurrence, and response to therapy. The biopsies were either embedded in OCT medium (Bayers Inc) or in paraffin wax. Mouse mammary gland tissue samples were obtained by removing the fourth inguinal gland from mice at various stages of postnatal development, i.e. from virgin, pregnant, lactating and regressing mice. The tissue specimens were fixed with 10% natural buffered formalin, dehydrated in a graded series of ethanols and xylenes, then embedded in paraffin wax. For immunohistochemical analysis, 6 μm sections were cut and mounted on poly-L-lysine coated slides.

**Antibodies and Immunohistochemistry**

Immunological reagents specific for each AP-2 family member were generated in our laboratory. Anti c-erbB-2 antibody was from Oncogene Science. During our previous studies we had raised a series of anti-peptide antisera and monoclonal antibodies which are specific for AP-2 α (13, 38). We have now produced anti-peptide antisera which are specific for either AP-2 β or AP-2 γ ( T. Williams, unpublished and 37). Antibodies used in our analysis were: monoclonal antibodies 3B5 and 5E4 specific for AP-2 α (original or 1:1 dilution); anti-peptide antisera β95 specific for AP-2 β (1:2000) and γ96 specific for AP-2 γ (1:200). The antibodies were diluted in 1xPBS containing 5% normal goat serum (Jackson Immuno-Research Laboratory) and 0.1% Tween 20 (Sigma). For immunohistochemical analysis on frozen sections, mounted sections were fixed in ice-cold acetone for 1 min, rinsed with 1xPBS twice. The slides were then blocked with 2% BSA in PBS for 30 min and 5% normal goat serum in PBS for another 30 min, followed by an 1 hour incubation at room temperature (or overnight incubation at 4°C) with the primary antibody. The slides were washed with PBS three times, 0.01% Triton X-100/PBS one time and PBS one time for 5 min each, followed by a 20 min application of secondary antibody, biotin-conjugated goat anti-mouse IgG (for the monoclonals), or goat anti-rabbit IgG (for the polyclonals) (1:250 dilution, Jackson Immuno-Research Laboratory). The slides were washed as above and then quenched in 1.0% $H_2O_2$/PBS for 10 min followed by rinsing with PBS. Then a 20 min application of streptavidin-peroxidase (1:500 dilution, Immuno-Research Laboratory) followed. After washing as above the staining was visualized by a 5-10 min incubation with a PBS solution containing 0.5 mg/ml 3,3'diaminobenzidine (Aldrich) and 0.01% $H_2O_2$. For staining on paraffin sections, slides were heated at 60°C overnight, deparaffinized with Xylene followed by ethanol, and then quenched with 2.5% $H_2O_2$ in methanol for 30 min. The slides were then re-hydrated with water followed by either blocking step as for frozen sections or treated with an additional antigen-retrieval procedure. The treatment is only needed for 3B5 and 5E4 antibodies. The procedure in as follows: a 10 mM citrate buffer, pH 6.0 with NaOH was heated till boiling in a pressure cooker. The slides were then placed in the boiling buffer. After closing the cover, the slides were cooked for about 5 min. The cooker was then depressed with tap cold water and the slides were allowed to cool to room temperature gradually and then rinsed with PBS. The slides were then ready for blocking step. For negative controls, each trial employed a slide in which a primary antibody was ommitted.

**Construction of Transgenes**

Transgenes were constructed using standard subcloning techniques. A FLAG epitope tag was attached to the 3' end of the AP-2 cDNA. This tag does not affect the function of the AP-2 protein as a DNA binding transcription factor (T. Williams, unpublished) and will enable us to distinguish between expression of the transgene and endogenous gene using an antibody specific for this novel FLAG epitope (available from Kodak). The fusion constructs of wild type or dominant negative forms of AP-2 were then placed under control of mouse mammary tumor virus long terminal repeat (MMTV LTR) using the plasmid pMSG (Pharmacia) which also contains a 3' SV 40

polyadenylation signal. The rat neu cDNA were placed under control of human c-erbB-2 promoter. Figure 1 shows the constructs for generating transgenic mice. Constructs A, B, C were digested with restriction enzymes Hind III and Xba I while constructs D was digested with Not I and Nhe I to remove vector sequences since those sequences are commonly believe to interfere with the transgene expression (29-31). The transgenes were then separated from the vectors by 10-40% sucrose density gradient ultracentrifugation. The fractions that contained transgene fragments were collected and dialyzed extensively against 10 mM Tris.Cl, 0.25 mM EDTA, pH7.5, and their concentration was adjusted to 1-3 μg/ml.

**Generation of Transgenic Mice**

Transgenic mice were generated by injecting DNA into pronuclei of fertilized eggs of inbred FVB mice (Taconic). The embryos surviving the microinjection were transferred into oviducts of pseudopregnant females (CD1 strain, Charles River). After birth, mice carrying the transgene were identified by Southern blotting using genomic DNA isolated from tail biopsies. The expression of transgenes were determined by RNase protection.

**Isolation of Genomic DNA and Southern Blot Analysis**

Genomic DNA was isolated from tails of 4-5 weeks old mice as described (40). 12 μg of genomic DNA was digested with appropriate restriction enzyme (construct A, D with Bgl II; C with Nco I and B with Acc 65I), electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose (Schleicher & Schuell) or Hybond N filters (Amersham). The filters were hybridized with probes derived from the transgenes which were either radioactively labeled by random-priming or labeled nonradioactively with "Genius Nonradioactive Nucleic Acid Labeling and detection Kit" (Boehringer-Mannheim). The hybridized products were visualized by autoradiography or by light-emission respectively.

**Isolation of RNA and RNase Protection**

Mouse mammary tissue was homogenized in guanidine isothiocyanate buffer and the total RNA was isolated as described (41). RNase protection was performed essentially as described (12), except that 10 μg of total RNA was used for each protection assay.

**Results**

**Immunohistchemical Analysis of Human Breast Cancer Biopsies**

We have recently established protocols which enable our specific antibodies to detect the various AP-2-related proteins on paraffin embedded or frozen sections from human breast cancer biopsies. Figure 2 and 3 show representative data obtained using these reagents. Figure 2A, 2E, and 3 show specific nuclear staining of tumor tissue from

three different patients obtained with 3B5 monoclonal antibody which is specific for AP-2 α. Data shown in Figure 2B ,C and D address the specificity of the antibody staining pattern. In particular, we have previously mapped the AP-2 epitope with which 3B5 reacts. The addition of a peptide corresponding to this epitope completely blocks reactivity (Figure 2B). Similarly, secondary antibody alone did not give the staining pattern (Figure 2C). However, a second monoclonal antibody, 5E4, directed against a different epitope within the AP-2 protein, produces an identical pattern of staining (Figure 2D). These data confirm that our monoclonal antibodies are producing bona fide signals corresponding to the presence of AP-2 α protein in breast cancer.

In human breast tumor biopsies apparently normal tissue can sometimes be found adjacent to a tumor. Data obtained from such "normal" samples should be interpreted with caution as they may be a connection between this "normal" tissue and the tumor. However, in the course of our analysis, we have documented the incidence of AP-2 expression in such apparently "normal" tissue. The preliminary studies indicated that the AP-2 protein can be observed in "normal" breast ductal epithelia (Figure 2F). However, expression was not observed in all samples (Figure 2G), and the intensity of staining was lower than that for tumor staining. Taken together, these data suggest that AP-2 may be present in the normal adult breast and also indicate that its expression may be differentially regulated.

We also compared the staining obtained with the AP-2 antibodies to that observed on adjacent sections with a commercially available monoclonal antibody directed against c-erbB-2. An example of the staining pattern observed in a breast cancer biopsy is shown in Figure 3. The presence of AP-2 detected using our monoclonal antibody is visualized by the brown nuclear staining, while the occurrence of the c-erbB-2 receptor is seen at the cell surface. This data clearly indicate the co-expression of c-erbB-2 and AP-2 in tumor tissue.

**Expression of AP-2 in Mouse Mammary Gland**

By using antibodies specific for each of AP-2 proteins we have performed immunohistochemical analysis on adult mouse mammary gland tissue from different developmental stages. Our preliminary experiments demonstrate that AP-2 proteins can be readily detected in the ductal epithelia of virgin and alveolar epithelial cells of pregnant mouse mammary gland. Figure 4 shows representative data obtained using 3B5 and γ96. The expression is shut off in the lactating mouse and re-activated during involution of mammary gland (data not shown). These studies suggest that AP-2 proteins have role in the growth and differentiation of mammary epithelia and will allow us to determine the correlation between the expression of AP-2, ER and c-erbB-2 throughout various stages of development of mammary gland.

**Generation of Transgenic Mouse Lines**

As shown above, AP-2 proteins are present in human breast tumors and are differentially regulated during the normal development of mouse mammary gland. We

wish to further understand the role of AP-2 in the development of the mammary gland and also in tumorigenesis. Therefore we are generating mouse model systems to determine the consequences of ectopic expression of AP-2 in the mouse mammary gland. To achieve these goals we have generated several transgenic mouse lines carrying different versions of the AP-2 gene. These mice will enable us to test the involvement of AP-2 in mammary gland development and its potential to induce mammary tumors either alone or in combination with c-erbB-2.

**A.** Generation of transgenic mouse lines with overexpression of wild-type AP-2 in the mammary gland

We have attached a FLAG epitope tag to the 3' end of the wild-type human AP-2 open reading frame. This fusion construct was placed under the control of the MMTV LTR, one of the best characterized regulatory moieties for directing expression to mouse mammary epithelia. The construct was microinjected into the pronuclei of one-cell mouse embryos. A total of ten transgenic founders were generated carrying the MMTV/AP-2 transgene. Of the ten lines, eight passed the transgene to their progeny in a Mendelian fashion. Since MMTV LTR-dependent gene expression is up-regulated by hormone changes during pregnancy, we have examined mice at this stage of maturation to assess the tissue-specificity of transgene expression. Total RNA was isolated from different tissues of 10-14 day pregnant females for each of transgenic lines and subjected to RNase protection with a transgene specific probe. Overexpression of the AP-2 transgene was noted in the mammary glands of three lines (J.Z. data not shown). Lower amounts of transgene transcript were also detected in lung, salivary gland and kidney in these three lines. We are breeding these transgenic lines to obtain a large enough colony so that we can analyze the consequences of this inappropriate expression of AP-2.

**B.** Generation of transgenic mouse lines carrying human c-erbB-2 promoter driven c-erbB-2 cDNA

We wish to determine if AP-2 can activate transcription of the c-erbB-2 gene in the mammary gland and consequently induce tumor formation. It has been reported that the rodent c-erbB-2 gene, also known as neu, is more efficient at tumor formation in the mouse than the human gene (6, 7). Therefore, I have now placed the rat homolog of the human c-erbB-2 gene under the control of human c-erbB-2 promoter (construct D). Transgenic mice have been generated using this construct and three founders can transmit the transgene to their progeny. We have now bred the transgenic mouse lines overexpressing wild-type AP-2 to the transgenic mice carrying transgene D (Figure 1). From the first set of breeding we obtained fourteen mice carrying both transgenes. These mice will be examined over a period of several months, and compared to their singly transgenic and non-transgenic normal relatives. We are breeding more of these transgenic mice.

C. Generation of transgenic mouse lines with overexpression of mutated forms of AP-2 in the mammary gland

Mutated forms of AP-2 either containing the entire DNA binding domain (Δ165) or only dimerization domain (Δ278) (13, 14) were tagged with the FLAG epitope and placed under the control of the MMTV LTR (Figure 1, constructs B and C). These constructs have now been used to derive their corresponding transgenic mice. So far, three founders were shown to carry transgene B and 8 founders to carry transgene C. We are breeding these founders now to check the transmission of transgenes and then we will determine the expression of transgenes as we did for the transgene A.

**Discussion**

Eukaryotic transcription factors are responsible for a variety of cellular processes during the development and maintenance of an organism. These proteins can form a regulatory cascade that is a fundamental mechanism for determination of cell fate. Because these molecules are such powerful regulators of genetic information, aberrant transcription factor activity can lead to developmental abnormalities and oncogenesis. AP-2 has now been shown to function in these normal and pathological processes. AP-2 has been implicated as a major determinant of cytokeratin and E-cadherin expression (44-46), and these cytoskeletal components have been detected by immuno-histochemistry in both normal and tumor tissue from the mammary gland (15, 48, 49). AP-2 has also been implicated in the control of ER expression. Taken together, AP-2 would therefore be expected to play a significant role in the establishment of the ductal network of the mammary gland and in the malignant development of breast tissue. Recently, AP-2 has also been shown to be involved in the c-erbB-2 overexpression. First, the promoter of the c-erbB-2 gene contains a critical AP-2 binding site that is responsible for overexpression in tumor cells. Second, the presence of AP-2 proteins correlates with overexpression of c-erbB-2 in breast cancer-derived cell lines. AP-2 has also been shown to activate transcription from the human c-erbB-2 promoter, and so it is likely that AP-2 is responsible for the inappropriate expression of c-erbB-2 associated with breast carcinogenesis. We wish to have a thorough understanding of the role of AP-2 in mammary gland development and mammary tumorigenesis.

In our preliminary studies we have performed immunohistochemical analysis of about 20 human breast cancer biopsies. These studies are very encouraging and suggest that AP-2 may provide an important reagent to probe breast cancer. First, the expression of AP-2 family members, especially α and γ, are observed in 80-90% of the samples so far analyzed. These in vivo findings show an excellent correlation with the data obtained from breast cancer cell lines. Second, c-erbB-2 expression is always associated with AP-2 expression. This again supports a role for AP-2 in the regulation of c-erbB-2 expression in the breast. Third, the prevalence of AP-2 staining in the tumors is greater than in the adjacent "normal" tissue. This finding suggests that the tumors are derived from a particular stage of ductal epithelium development or that the expression of AP-2 is reactivated in the tumors. Fourth, AP-2 staining was not observed in other common solid tumors, which were derived from lung or colon tissue

(data not shown). This finding indicates that AP-2 is not a general marker of tumor tissue. Moreover, it suggests that AP-2 may be a useful diagnostic reagent to identify metastatic breast cancer in these organs. The ability to distinguish between a metastatic breast cancer or a novel primary tumor, derived from lung or colon, would have profound implications for patient therapy.

The presence of AP-2 in the tumors could result from the escape of a particular cell-type from normal cell-cycle regulation, or represent the reactivation of an earlier developmental pathway. The most direct method to address this issue is to examine AP-2 expression throughout the normal development of human mammary gland. However, this tissue is not always readily available. Mouse mammary gland tissue, in contrast, can be readily obtained and analyzed at a variety of developmental stages and thus provide a powerful experimental system to examine and manipulate breast development. We have determined the expression of AP-2 proteins throughout postnatal mouse mammary gland development by immunohistochemistry. Our studies shown that AP-2 can readily detected in ductal epithelial cells in virgin mice and lobuloalveolar epithelial cells in pregnant mice. The expression is down-regulated to an undetectable level during lactation. These data suggest the following model for the role of AP-2 in mammary gland cell-fate determination. The stages of maturation when AP-2 is observed correspond to the timepoints when there is marked proliferation of the ductal network. Conversely, when the cells stop dividing and assume their terminal state of differentiation, no significant levels of AP-2 can be observed. Taken together, these findings indicate that AP-2 may be involved in mammary gland proliferation and the early stages of differentiation into the lobuloaveolar network. Another interesting aspect is that AP-2 is reactivated during involution. At this stage, the mammary gland undergoes extensive remodeling, leading to the loss of the alveolar structures, in a process involving large scale apoptosis (42, 43). It will be interesting to determine if AP-2 marks the cells that are targeted to survive, or undergo apoptosis, or both, during this stage. These experiments provide a basis for understanding the importance of AP-2 in growth, maturation and remodeling of mammary gland and will help us understand the role of inappropriate expression of AP-2 in the transgenic mice of the following experiments and in tumorigenesis.

We are now directly testing the involvement of AP-2 in mammary gland development and tumorigenesis using molecular genetic approaches. In particular, we have generated transgenic mouse lines in which expression of either wild-type or dominant negative forms of AP-2 have been targeted to the mammary gland using the MMTV LTR. We have obtained three lines which overexpress wild-type AP-2 in the mammary gland. Given the association of the various AP-2 proteins with overexpression of ER and c-erbB-2 in a number of breast cancer cell lines, it will be of interest to determine if overexpression of this exogenous AP-2 transgene can activate the endogenous mouse ER and c-erbB-2 genes. If inappropriately expressed AP-2 can activate the endogenous mouse genes then this might result in mammary tumors. In addition, because AP-2 has been shown to possess some ability to convert cells to anchorage independent growth in tissue culture (32), it is possible that it will be able to

participate in tumorigenesis independent of c-erbB-2. We are now monitoring these mice for any sign of tumor formation occurring.

AP-2 gene is a critical regulator of many aspects of mammalian embryogenesis (38, 39) including the tissue which gives rise to the earliest stage of the foetal mammary gland. It is therefore important to determine the consequences of removing this gene with respect to the development of the mammary gland. We can not perform these studies on our knockout mice since the mice die at birth and the body wall, where the mammary rudiments reside, is disrupted. Fortunately, the AP-2 gene family offers a suitable target for an alternative dominant negative strategy. In particular, there is considerable amino acid sequence conservation between all three AP-2 family members. One consequence of this similarity is that these proteins all recognize the same DNA binding site. A second important observation is that these proteins can all form heterodimers (37). Thus dominant negative proteins which influence DNA binding or dimerization should affect all AP-2 family members concurrently. We have generated transgenic mice with these mutated forms (either DNA binding domain or dimerization domain). We are breeding these founders and will soon determine the expression of these mutants in the offspring. Subsequently, the effects of the dominant negatives on the development of the mammary gland will be analyzed.

Finally, we would like to test if the co-operation of AP-2 and c-erbB-2 could induce tumor formation in mammary gland. We have generated transgenic mice in which the human c-erbB-2 promoter drives the rat c-erbB-2 cDNA. We have bred these mice with the mice overexpressing AP-2 in the mammary gland. We are now in the process of determining the consequences of the co-expression of these two transgenes on mammary gland pathogenesis. The phenotype of these doubly transgenic mice will be compared with those of singly transgenic or nontransgenic littermates. If tumors occur, the animals will then facilitate the analysis of the mechanism involved in the early stages of tumorigenesis which is critical for the detection and treatment of breast cancer. Furthermore, we can bred these doubly transgenic mice with the mice carrying the dominant negative forms of AP-2 and test if the dominant negatives can neutralize the function of wild-type AP-2 and consequently reduce the incidence of tumors.

In conclusion, our data strongly suggest a role for AP-2 in controlling gene expression in both normal mammary gland development and also in breast cancer. We are now directly addressing the involvement of AP-2 in mammary gland development and tumorigenesis using animal model systems. We have made significant progress toward the goal of understanding the role of AP-2 in breast development and breast cancer. These studies will provide insight into the etiology of breast cancer and allow an assessment of the value and feasibility of using the AP-2 proteins as targets for gene therapy.

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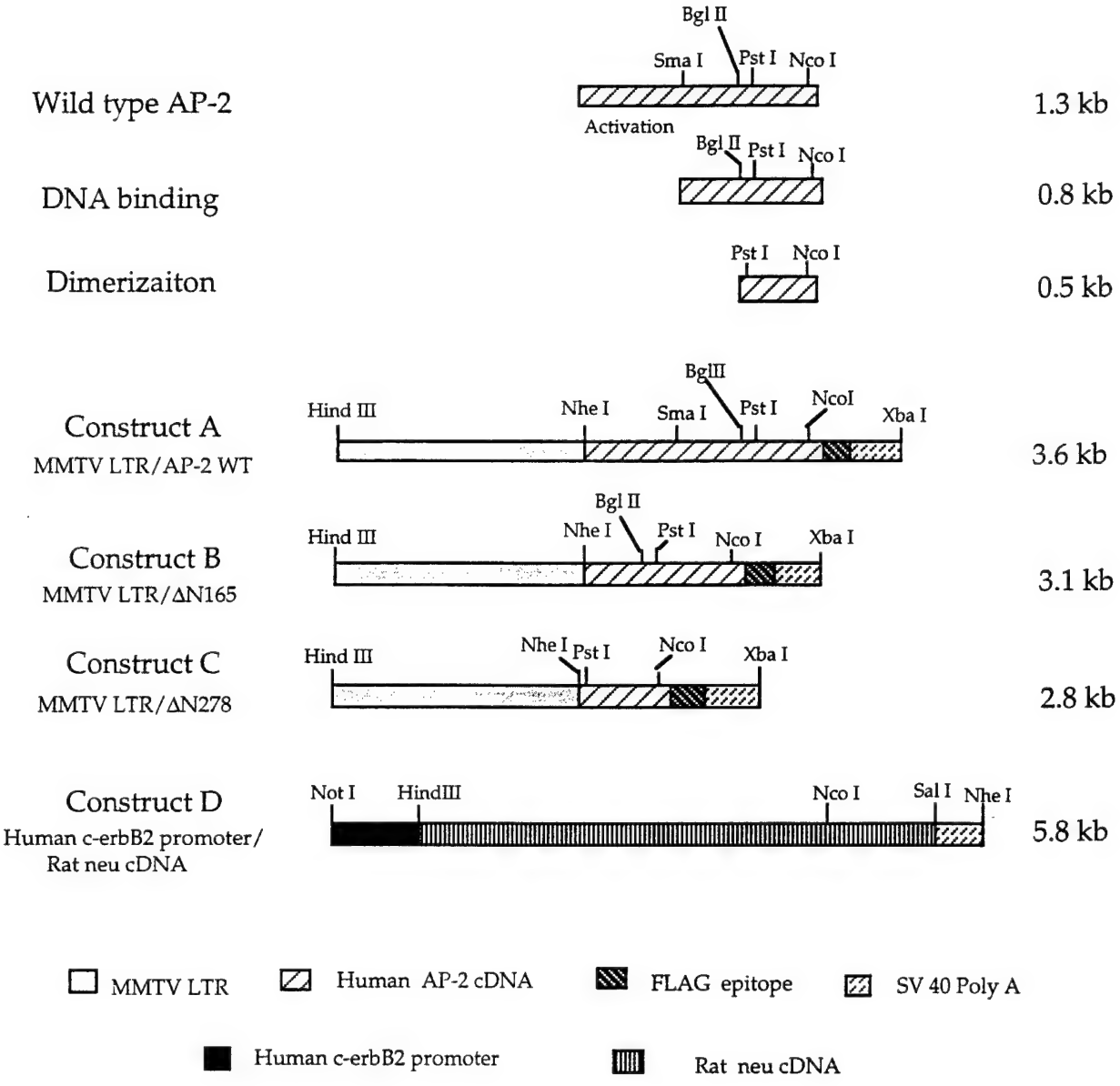


**Figure 1.** Constructs for generating transgenic mice and partial restriction maps of the constructs. The drawing is not to scale.

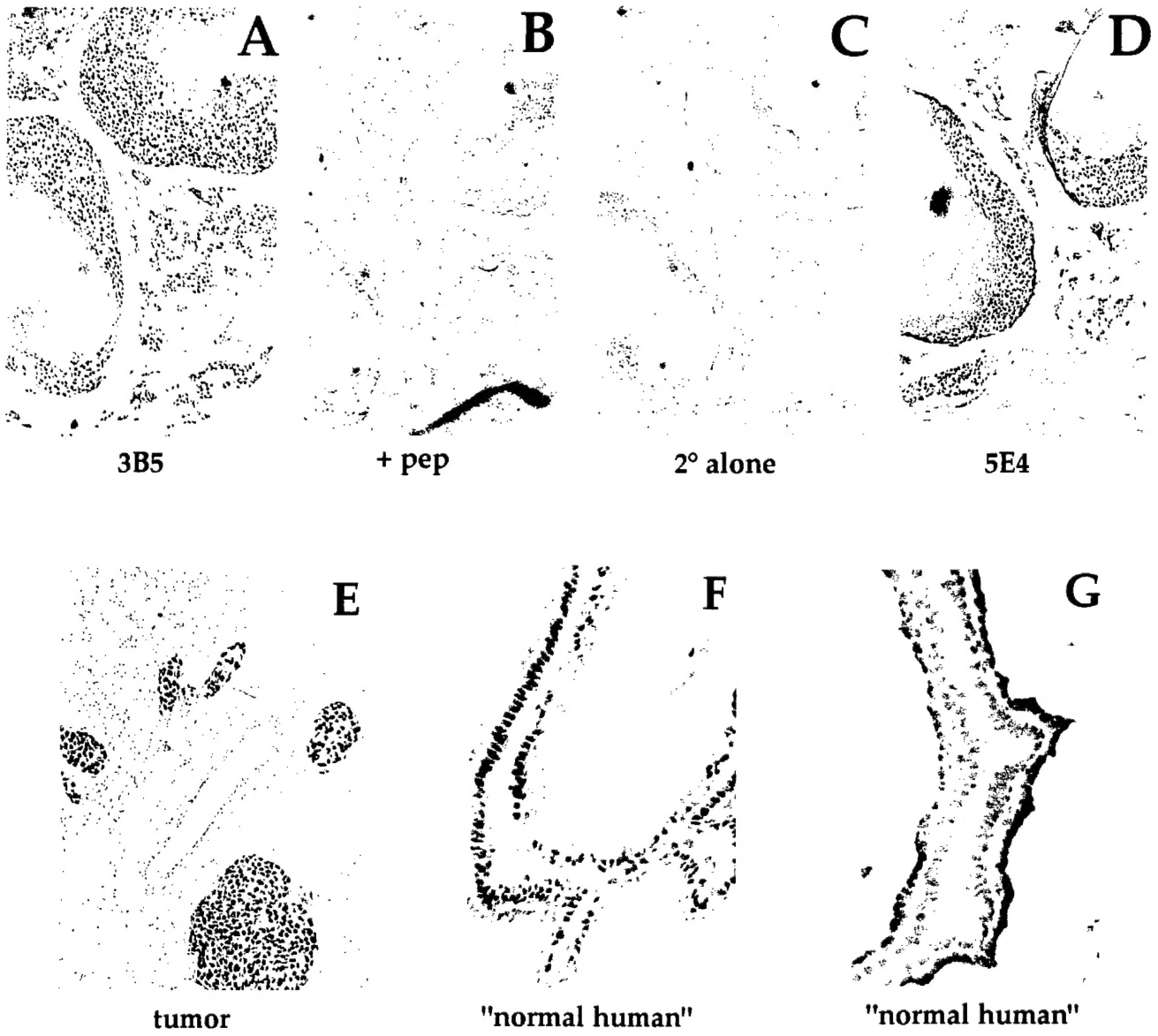


**Figure 2. Expression of AP-2 in human breast cancer biopsies.**

**A.** Detection of AP-2 protein in human tumor sample #5668 using mouse monoclonal antibody 3B5. **B.** The tumor staining obtained with 3B5 is blocked by the addition of a specific peptide equivalent to the epitope recognized by this antibody. **C.** No specific staining is observed when the primary 3B5 antibody is omitted. **D.** A second mouse monoclonal antibody, 5E4, which recognizes a distinct AP-2 epitope from 3B5 produces the same pattern of nuclear staining in an adjacent section. **E.** Staining of a second human tumor sample, #11575, with 3B5. **F.** Staining of a "normal" duct from tumor sample #22577 with 3B5. **G.** The "normal" duct from tumor sample #5470 does not stain with 3B5. Note that adjacent tumor tissue did stain with this antibody (not shown).

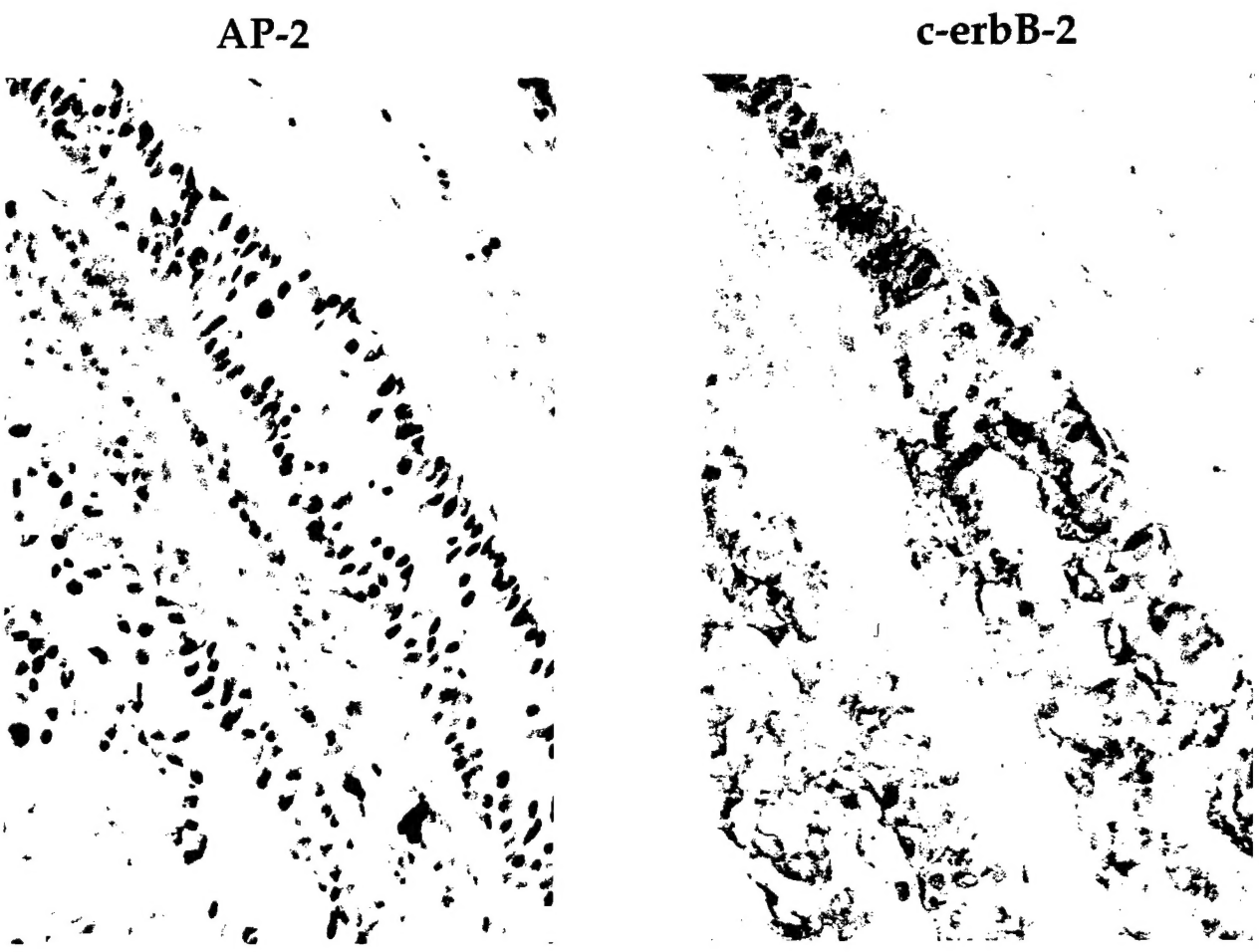


**Figure 3. Co-incidence of AP-2 and c-erbB-2 expression in a human breast biopsy #21114.**

The AP-2 staining with the monoclonal 3B5 is seen as brown nuclear staining as expected for a transcription factor. In contrast, the c-erbB-2 staining is observed at the cell surface consistent with the function of this protein as a cell surface receptor.

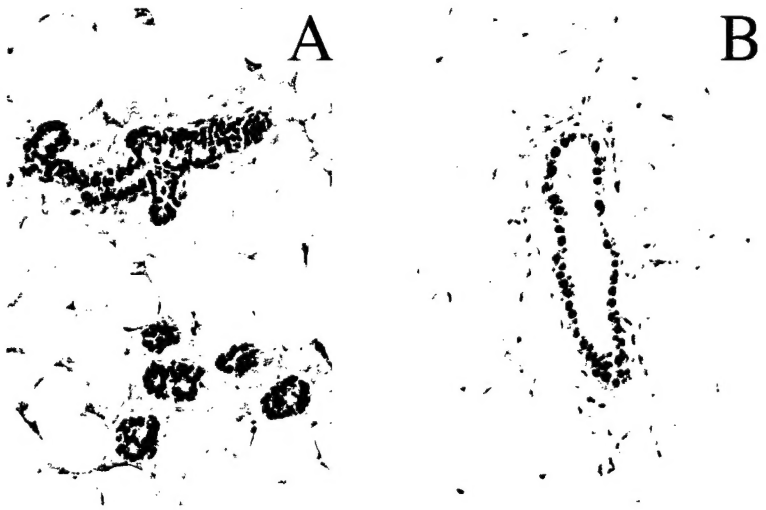


**Figure 4. Expression of AP-2 transcription factor family in the normal mouse mammary gland.**

**A.** Staining of normal mammary gland tissue from a 14 days pregnant mouse with 3B5.
**B.** Staining of normal mammary gland tissue from a 7 weeks old virgin mouse with γ96.

# ANIMAL USE REPORTING

Activity Name & Address: Jian Zhang, Dept. of Biology, Yale University
266 Whitney Ave. New Haven, CT 06520

Contract number: DAMD17-96-1-6094

| Animal Type Genus/Species | Animals Purchased or Bred | Animals Used | USDA Pain Column C | USDA Pain Column D | USDA Pain Column E | AAALAC Accreditation |
|---|---|---|---|---|---|---|
| FVB | 900 | 750 | no pain 800 | moderate pain 100 | | |
| CD1 | 200 | 180 | no pain 20 | moderate pain 180 | | |
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